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Antimicrobial activity in sub-Arctic marine invertebrates

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Abstract In the marine environment, any living or non-living surface is exposed to bacterial colonization. Many invertebrate species in temperate, tropical and Antarctic regions have demonstrated chemical defences against the formation of microbial films. In the present study, the antimicrobial activity of sub-Arctic invertebrates was investigated for the first time. Crude extracts of abundant invertebrates belonging to several taxonomic groups were tested for their inhibitory effects on the growth of five sympatric phylogenetically diverse bacterial strains. Six out of 18 (33%) crude extracts inhibited bacterial growth at natural extract concentrations. The crude extract of the sponge *Haliclona viscosa* inhibited growth of all five bacterial strains, suggesting the presence of metabolites with broad-spectrum activity. Three active compounds were isolated from *H. viscosa* having antibacterial properties similar to those of the crude extract. Our data indicate that antibacterial secondary metabolites are present in sub-Arctic marine invertebrates but are less abundant than in temperate, tropical or Antarctic species.

Introduction

The flora and fauna in the marine environment are virtually bathed in a ''microbial soup'' (Jenkins et al. 1998), and any solid living or non-living surface is exposed to colonization by bacteria. Formation of so-called microbial biofilms is initiated by the adsorption of macromolecules to a surface immediately after contact with seawater, followed by adsorption and adhesion of bacteria (Davis et al. 1989; Wahl 1989). Bacterial colonization and surface conditioning have been considered to be the first stages of surface fouling, promoting subsequent settlement of unicellular and multicellular eukaryotic organisms (Wahl 1989). Filter-feeding animals, such as sponges, that feed on bacteria (Bergquist 1978) concentrate micro-organisms from the water column during the feeding process and are thus additionally exposed to high quantities of microbes, including potential pathogens. However, the incidence of infection appears to be relatively low in nature, considering the permanent presence of all types of bacteria (Jenkins et al. 1998). Bacteria often do not colonize marine organisms uniformly, since microbial colonization can be reduced by mechanical processes like tissue or mucus sloughing (Krupp 1985; Barthel and Wolfrath 1989), by physical properties like surface tension (Becker and Wahl 1991), by surface acidity (Hirose et al. 2001) and also by secondary metabolites (McCaffrey and Endean 1985; Wahl et al. 1994; Henrikson and Pawlik 1995; Newbold et al. 1999).

Studies on the chemical antibacterial properties of invertebrates cover a wide range of taxonomic groups and geographic regions. Investigations addressing geographical variations often propose chemical defences to be increased in tropical organisms compared to temperate species (Bakus 1974; Bakus and Green 1974; Green 1977; Hay and Fenical 1988; Bolser and Hay 1996). However, McCaffrey and Endean (1985) found antimicrobial activity in tropical and sub-tropical sponges from the Pacific to be similar to that in organisms from temperate regions. More recently, some investigations included chemical defences against surface bacteria in polar waters (McClintock 1987; Slattery et al. 1995). McClintock and Gauthier (1992) found antimicrobial properties in Antarctic sponges to be widespread although the activity was generally weaker compared to temperate and tropical species, indicating a latitudinal decline of activity. So far,

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little information on latitudinal variation in antimicrobial chemical defences of marine invertebrates is available from Arctic waters.

It could be hypothesized that organisms would invest more resources into antimicrobial defence when the threat of bacterial colonization is higher. Although bacterial numbers alone do not necessarily reflect the selective pressure exerted on marine invertebrates, it could be one indication of the potential of detrimental surface colonization or infection. Total bacterial numbers in cold waters are proposed to be generally decreased compared to lower latitudes. In the Beaufort Sea, for example, $0.2–0.9\times10^6$ bacteria per millilitre are found (Atlas and Morita 1986), similar to $<0.37\times10^6$ ml^{-1} in Antarctic waters (Zdanowski 1995). Bacterial abundance is higher by almost an order of magnitude in the Baltic Sea (10.9×10^6 ml^{-1}, Lignell et al. 1992), in the Atlantic (2.6×10^6 ml^{-1}, Hanson et al. 1988), and the Pacific Ocean ($<5\times10^6$ ml^{-1}, McManus and Peterson 1988). Locally, however, bacterial numbers can be increased up to 8×10^6 ml^{-1} at Arctic inner-fjord locations, due to a high amount of particulate organic carbon at glacial meltwater outflows (K. Jankowska and M. Wlodarska-Kowalczuk, unpublished work).

The aim of the present study was to assess, for the first time, the chemical antimicrobial properties of various invertebrates in a fjord at Spitsbergen. Effects of crude extracts, fractions and pure compounds of high-latitude invertebrates on sympatric micro-organisms were tested. We screened a broad spectrum of abundant marine invertebrate taxa from Kongsfjord, including sponges, actinians, soft corals, molluscs, bryozoans and ascidians for antibacterial activity.

Materials and methods

Study site

Our study was performed at Kongsfjord, situated at the north-western coast of Spitsbergen (79°N, 12°E). A comprehensive overview of the present biological and physical knowledge of the fjord is provided in two recent reviews (Hop et al. 2002; Svendsen et al. 2002). The fjord extends 26 km landwards with a width of 3–8 km and a maximum depth of about 400 m. Despite the high-latitudinal location, the fjord has sub-Arctic rather than Arctic properties, due to the strong influence of relatively warm Atlantic water masses at the northwestern coast of Spitsbergen (Svendsen et al. 2002). The summer season is characterized by considerable glacial activity, with four glaciers and several river run-offs adding high loads of terrestrial sediments and freshwater to the fjord (Elverhoi et al. 1983; Weslawski et al. 1995). The annual mean water temperature is slightly above 0°C (Ito and Kudoh 1997); however, in summer, maximum temperatures of about 6°C at the surface and about 4°C at 20 m depth were measured (Hanelt et al. 2001). Invertebrates were collected at four different sites in the fjord. While sites 1, 3 and 2 (Hansneset, a cave close to Hansneset, and Kongsfjordneset, Fig. 1) are dominated by hard-bottom communities, site 4 (Prinz Heinrich Islands, Fig. 1) is characterized by soft sediments with single stones and boulders, forming patches of hard-substrate habitats.

Sampling

Seventeen species of sessile or slow-moving invertebrates that were abundant at the different sampling sites, and the egg mass of a gastropod were collected by scuba-divers in the summers of 1999, 2000 and 2001. Samples were immediately brought to the laboratory and epibiontic organisms, when present, were removed manually from the surfaces. After gently blotting with tissue paper to remove extracellular water, samples of each species were weighed, shock-frozen in liquid nitrogen and lyophilized. The freeze-dried tissue was weighed again and stored at −28°C until extraction. For later species identification, voucher specimens were preserved in a 5% (v/v) formaldehyde-seawater solution. Information on the taxa studied, the respective collection sites and depth of sampling is given in Table 1. Taxonomic identification of the ascidian *Styela* spp. showed that the collected material consisted of a mixture of the two morphologically similar species *Styela rustica* and *S. gelatinosa*.

Extraction procedure

A known mass of freeze-dried tissue from each species was ground with mortar and pestle and subsequently extracted in 100% methanol, 1:1 (v/v) methanol:dichloromethane, and 100% dichloromethane, or repeatedly in 1:1 (v/v) methanol:dichloromethane under permanent stirring on a magnetic stirrer at room temperature. The partial extracts were combined (see Table 1 for details)

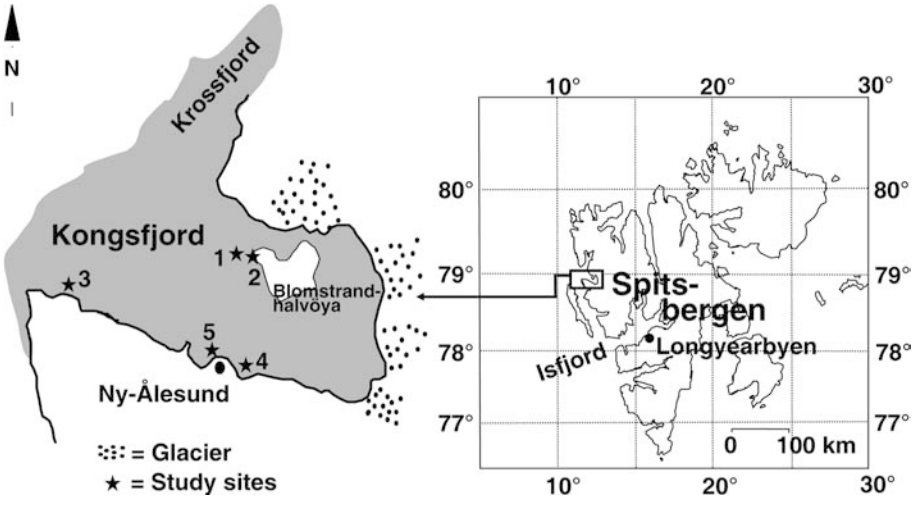

Fig. 1 General view of Spitsbergen, showing Kongsfjord and the study sites. *Asterisks* and *numbers* indicate the different sampling sites (*1–4*): *1* Hansneset; *2* Cave; *3* Kongsfjordneset, *4* Prinz Heinrich Islands

Table 1 Investigated invertebrate species, sampling site and water depth of sampling, extraction method and estimated natural concentration of crude extracts. Extraction method *A* indicates extraction in 1:1 MeOH/DCM; method *B* means subsequent extraction in 100% MeOH, 1:1 MeOH/DCM, 100% DCM. Natural concentrations are given in mg extract/g WW (wet weight)

	Taxa	Collection site	Depth of collection (m)	Extraction method	Natural concentration (mg/WW)
Porifera	*Haliclona viscosa*	Hansneset	15–25	B	36.3
	Haliclona rosea	Hansneset	10–25	B	18.8
	Suberites ficus	Cave	4–5	A	46.3
	Spongosorites genitrix	Cave	4–5	A	38.6
Cnidaria	*Hormathia nodosa*	Hansneset	10–25	A	34.9
	Urticina eques	Kongsfjordneset	10–25	A	27.7
	Urticina asiatica	Kongsfjordneset	10–25	A	19.3
	Gersemia rubiformis	Cave	2–4	A	44.1
Bryozoa	*Tricellaria ternata*	Hansneset	1–10	A	38.2
	Eucratea loricata	Hansneset	1–6	B	26.2
	Crisiella sp.	Hansneset	1–6	A	43.8
	Alcyonidium gelatinosum	Hansneset	1–8	A	29.6
Gastropoda	*Natica* sp. (egg mass)	Various sites	4–30	A	10.2
Nudibranchia	*Dendronotus frondosus*	Various sites	1–25	A	39.5
	Flabellina salmonacea	Various sites	1–25	A	30.3
Ascidiacea	*Halocynthia pyriformis*	Hansneset	5–25	B	25.8
	Styela spp.(*S. gelatinosa*, *S. rustica*)	Prinz Heinrich Islands	15–30	B	45.4
	Synoicum turgens	Hansneset	1–7	A	13.1

and filtered to remove particles, followed by concentration under reduced pressure using a rotary evaporator (40°C). Crude extracts were transferred into pre-weighted vials, evaporated to dryness under nitrogen or vacuum, and weighed. The extract yield per g WW (Table 1) is referred to as the natural concentration. This is the most widely used approach but it may not always reflect the actual concentration at an organism's surface in nature (Pawlik et al. 1988; Becerro et al. 1997; Schupp et al. 1999), and slight overas well as under-estimations may be possible. Attempts to elucidate actual surface concentrations of antifouling compounds have only recently begun for macroalgae (de Nys et al. 1998) and more research is necessary to develop this methodology further. To obtain sufficient mass of crude extracts, we used pooled samples of several individuals. All extracts were stored at −28°C until use in antibacterial experiments.

Crude extracts of *Haliclona viscosa* and *Halocynthia pyriformis*, both exhibiting antibacterial activity, were further partitioned with *n*-hexane. The aqueous phase was dried and re-dissolved in water, followed by subsequently partitioning with ethyl acetate (EtOAc) and butanol (*n*-BuOH). The EtOAc and *n*-BuOH fractions of *Haliclona viscosa* were combined after HPLC analysis revealed no differences in their chemical composition. The remaining water fractions of both species were also subjected to bioassays. The active EtOAc/*n*-BuOH fractions of *Haliclona viscosa* were further purified with low-pressure liquid chromatography (silica gel, hexane:EtOAc 6.5:3.5) and preparative RP-HPLC using a Kromasil RP 18 column (4.6×250 mm, 5 µm particle size), respectively.

Bacterial isolation and sequence analysis

Bacterial strains from the vicinity of the investigated invertebrates were isolated from stones, sediment and seawater from about 12 m water depth at site 1 (Fig. 1.) These samples were collected using sterile plastic bags, and were immediately transferred to the laboratory. Bacteria were obtained from stones by swabbing the surface with a sterile cotton tip followed by inoculation of agar plates. Sediments were suspended in 10 times volume of sterile seawater, allowed to settle and 100 µl of the supernatant was used for inoculation of agar plates using a sterile glass rod. From seawater samples, 100-µl aliquots were directly spread on agar plates. We used marine agar medium after Zobell (1941) in two different nutritional concentrations, including a low-nutrient agar that may better reflect the nutrients available to marine bacteria in their natural environment (Zobell I: 94% seawater, 5% peptone, 0.9% yeast extract, 0.1% $FePO_4$; Zobell II: 99.4% seawater, 0.5% peptone, 0.1% yeast extract, 0.01% $FePO_4$). Bacteria were incubated at 4°C, which approximately equals seawater temperature in the Kongsfjord during summer. Numerous pure cultures were established from the different substrates.

From all cultures, five bacterial strains (Table 2) were chosen and identified by sequence analysis of 16S rDNA. Nucleic acids were extracted from the isolated cultures with the DNeasy Tissue Kit (Qiagen, Hilden, Germany). From template DNAs (approx. 100 ng), 16S rDNAs were amplified by hotstart PCR (Saiki et al. 1988) with an automated thermal cycler (Eppendorf, Germany) using the bacterial-specific primers 8*f* (5′-AGA GTT TGA TCM TGG C-3′) (Giovannoni 1991) and 1492*r* (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Lane 1991) as follows: 50 pmol of each primer, 2.5 µmol of each deoxyribonucleoside triphosphate, 1×PCR buffer, 40 µl Taq Master enhancer (pre-heated, Eppendorf) were adjusted to 100 µl with sterile water. The addition of Taq Master to the reaction mixture improved the success of the PCRs. Master Taq (1 U, Eppendorf) was added to the reaction mixture at 60°C after an initial 5 min denaturation step at 95°C. The cycles were as follows: 30 cycles at 95°C for 1 min, 45°C for 1 min, and 72°C for 3 min, and a final elongation step at 72°C for 10 min. PCR products were purified using the QIAquick Purification Kit (Qiagen). 16S rDNAs were sequenced with an ABI PRISM 3700 capillary sequencer (Applied Biosystems, Foster City, Calif.) using AmpliTaq DNA Polymerase and 16S rDNA specific primers. Sequence data were analysed with the ARB software package

Table 2 Bacterial strains isolated from the vicinity of the investigated invertebrates

Bacterial strain and accession number	Isolated from	Medium	Gram staining
A (AY198113)	Water column	Zobell I	Negative
B (AY198114)	Stone	Zobell I	Positive
C (AY198115)	Stone	Zobell I	Negative
D (AY198116)	Sediment	Zobell I	Negative
E (AY198117)	Water column	Zobell II	Negative

(http://www.mikro.biologie.tu-muenchen.de) and a phylogenetic tree was reconstructed using maximum-likelihood analyses. Only 16S rDNA sequences containing at least 1,400 bases were used for tree construction. Filters for phylogenetic subdivisions and/or groups that considered only 50% conserved regions were applied to exclude highly variable positions.

The 16S rRNA sequences generated in this study were deposited in the GenBank database (Benson et al. 1999) under the following accession numbers: isolate A, AY198113; isolate B, AY198114; isolate C, AY198115; isolate D, AY198116; and isolate E, AY198117.

Antimicrobial assay

The agar disc-diffusion assay (Acar 1980) was used to test for antibacterial activity of extracts of 17 invertebrate species and the egg mass of a gastropod (Table 1) against the 5 sympatric bacterial strains. To guarantee high cell densities, bacterial strains were grown in liquid medium (99.4% seawater, 0.5% peptone, 0.1% yeast extract, 0.001% $FePO_4$) at 4°C for at least 7 days prior to the experiments. Inocula of 200 µl of each strain were then spread on separate agar plates (99.4% seawater, 0.5% peptone, 0.1% yeast extract, 0.001% $FePO_4$) using a sterile glass rod to provide a uniform film of the test bacteria.

Crude extracts and fractions were dissolved in aliquots of the extraction solvent to give natural concentrations (Table 1). Pure compounds were brought up in concentrations of 5 mg/ml. Since none of the three compounds isolated from the EtOAc/*n*-BuOH fraction of the sponge *Haliclona viscosa* (X2, X3, X4) exhibited antibacterial effects comparable in strength to the effects caused by the EtOAc/*n*-BuOH fraction, these compounds were recombined to test for synergistic effects. Compounds for recombination were used in the respective proportions they contributed to the total yield of the EtOAc/*n*-BuOH fraction.

We applied 10 µl crude extract, fraction or pure compound onto each side of a sterile paper disc (Ø 6 mm, Whatman). The volume added represents the approximate volumetric capacity of the discs. Discs were then placed in a previously sterilized (160°C) drying oven at 30°C, and solvents were allowed to evaporate. Control discs were prepared in the same manner with solvent only. Up to six extract discs and one solvent control disc per plate were placed on the surface of agar plates previously seeded with individual bacterial strains. According to extract availability, one to five replicates per extract were tested. The radius of the inhibition zone (without disc) was measured to the nearest 0.5 mm following incubation at 4°C over 5 days. Solvent control discs were never observed to inhibit bacterial growth. Since bacterial films of strains B and C were still very transparent after 5 days and zones of inhibition were difficult to distinguish, we measured inhibition zones again after 6 additional days for all five strains. Only minor differences in size of inhibition zones were found between measurements after 5 and 11 days. Results were categorized as no effect (0), weak inhibition (0–1 mm), moderate inhibition (>1 to 3 mm), strong inhibition (>3 to7 mm) and very strong inhibition (>7 to 15 mm). Due to the quantity of extract available, the number of replicates varied between one and six.

Results

Identification of bacterial strains

Bacterial isolates were phylogenetically diverse and clustered within clades of previously cultured bacteria from polar oceans or sea ice (Fig. 2). Isolates A, C and D were most closely related to *Psychrobacter* spp., *Colwellia* spp. and *Pseudoalteromonas* spp., respectively, within the γ-proteobacteria. Isolate E, within the *Cytophaga*/*Flavobacterium* (C/F) group, associated with *Polaribacter* spp., and isolate B, a Gram-positive bacterium, associated with *Planococcus* spp.

Antibacterial tests

Results of antimicrobial experiments testing crude extracts of 17 invertebrate species and of the egg mass of a gastropod against 5 strains of sympatric bacteria are shown in Table 3. Antibacterial effects differed with respect to extracts and bacterial strains. Six out of 18 extracts inhibited growth of at least 1 bacterial strain at natural extract concentrations. The extract of the sponge *Haliclona viscosa* had the strongest antimicrobial activity in terms of the number of strains inhibited and the radius of the inhibition zones. Only *Haliclona viscosa* inhibited the growth of all five test bacteria, while the soft coral *Gersemia rubiformis*, the bryozoan *Alcyonidium gelatinosum*, and the nudibranch *Flabellina salmonacea* inhibited two strains; the bryozoan *Crisiella* sp. and the ascidian *Halocynthia pyriformis* both inhibited one strain. The two bacterial isolates most frequently inhibited were strains B and C.

Crude extracts of the soft coral *G. rubiformis* and the ascidians *Halocynthia pyriformis* and *Styela* spp. were additionally tested at fivefold natural concentrations (Table 3). This increase in concentration caused an increase in inhibition zones of strains B and C in response to the extract of *G. rubiformis*, as well as an inhibition of two additional strains (B and E) by the extract of *Halocynthia pyriformis*. The extract of *Styela* sp. did not have any effect on bacterial growth at natural concentration, but had slightly inhibiting effects on strain B at fivefold natural concentration.

Active crude extracts of *Haliclona viscosa* and *Halocynthia pyriformis* were further partitioned and antimicrobial activity of fractions was tested in the agar disc-diffusion assay. The crude extract of *Haliclona viscosa* was separated into three fractions, yielding an *n*-hexane, an EtOAc/*n*-BuOH and an aqueous remain fraction. EtOAc, *n*-BuOH, *n*-hexane and water fractions were obtained from the crude extract of *Halocynthia pyriformis*. The EtOAc/*n*-BuOH soluble compounds from *Haliclona viscosa* and the EtOAc fraction from *Halocynthia pyriformis* inhibited bacterial growth, whereas all other fractions of both species had no effect (Table 4). Antibacterial activity of the EtOAc fraction of *Halocynthia pyriformis* was very low at natural concentration (<1 mm, strain B) but increased at fivefold natural concentration (1–2 mm, strains B and C). Growth of strain E was not affected by the EtOAc fraction, although the crude extract showed weak growth inhibition of this bacterial strain.

Further purification of the active *n*-BuOH/EtOAc fraction of *Haliclona viscosa* resulted in three pure compounds, X2, X3 and X4. Structural analysis of these compounds is still ongoing but preliminary results show

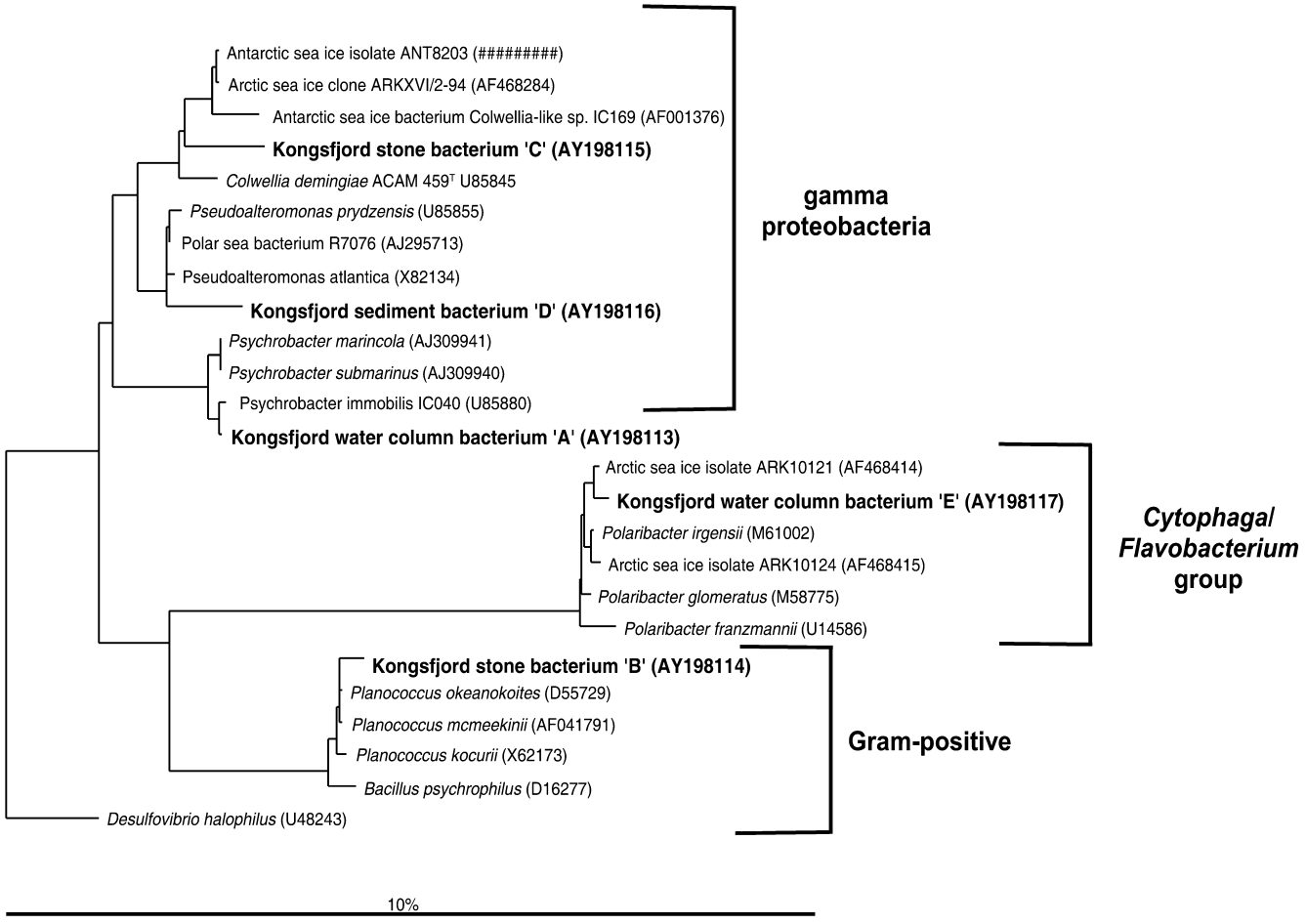

Fig. 2 Phylogenetic tree based upon maximum likelihood (FastDNAml) analysis. Outgroup is *Desulfovibrio halophilus* (U48243). The *scale bar* indicates 10% estimated sequence divergence. The lengths of *vertical lines* are not significant. Isolated strains "A", "B", "C", "D", "E" are in **bold type**

that they are related to the alkaloids cyclostelletamine (Fusetani et al. 1994) and haliclamine (Fusetani et al. 1989; C. Volk, H. Lippert, M. Köck, unpublished work). Results of all *Haliclona viscosa* fractions and compounds are given in Table 4. Inhibition of bacterial growth by the crude extract was stronger (five strains, strong to very strong inhibition) than by the *n*-BuOH/EtOAc-fraction or by the pure compounds. The *n*-BuOH/EtOAc fraction inhibited growth of all bacterial strains but effects were weaker (moderate to strong) than in crude extract. Purified compounds X3 and X4 exhibited strong to very strong inhibition of test strains B and E while there was no effect on strains A and D, and only a weak effect of X4 on strain C (< 1 mm) (Table 4). Compound X2 had a moderate inhibitory effect on strains A, B and D and a strong effect on strain E, but did not affect strain C. Since none of the effects caused by the individual compounds, X2, X3 and X4, corresponded exactly with the antibacterial effects caused by the crude extract or the *n*-BuOH/EtOAc fraction, we also conducted experiments with all possible recombinations of the three compounds, but did not find any synergistic or additive effects (Table 4).

Discussion

Bacterial test strains

The bacteria isolated from Kongsfjord and employed in the agar disc-diffusion assays were typical cultivable members of the marine microbial community in cold seas. Members of the genus *Polaribacter* (isolate E) are ubiquitous in polar oceans (Gosink et al. 1998) and several of the isolates (A, B, D) are representative of species associated with surfaces in the marine environment. *Psychrobacter* spp. and *Pseudoalteromonas* spp., as well as Gram-positive bacteria, have been found to be associated, for example, with bryozoans in the North Sea (Pukall et al. 2001). The five bacterial strains used here in antibacterial assays thus represent a diverse array of marine bacteria (Fig. 2). They have the potential to come into contact with the invertebrates tested, although we do not know if they would affect these invertebrates positively or negatively. As discussed by Jenkins et al. (1998), ecologically sensible bioassays have to involve the use of micro-organisms that interact with the investigated organism in nature. It has been shown that

Table 3 Inhibition of bacterial growth by crude extracts of 18 invertebrate species. Radius of inhibition zone: 0 no effect; 0–1 mm *X* (weak inhibition); >1–3 mm *XX* (moderate inhibition); >3–7 mm *XXX* (strong inhibition); >7–15 mm *XXXX* (very strong inhibition)

Species	Replicate	Bacterial Strain					∑ strains inhibited
		A	B	C	D	E	
Porifera							
Haliclona viscosa	1	XXX	XXXX	XXXX	XXX	XXXX	5
Haliclona rosea	1	0	0	0	n.d.	n.d.	–
	2	0	0	0	0	0	0
Suberites ficus	1	0	0	0	n.d.	n.d.	–
	2	0	0	0	0	0	0
Spongosorites genitrix	1	0	0	0	0	0	0
Cnidaria							
Hormathia nodosa	1	0	0	0	0	0	0
Urticina eques	1	0	0	0	0	0	0
Urticina asiatica	1	0	0	0	0	0	0
Gersemia rubiformis	1	0	X	XXX	0	0	2
Fivefold	1	0	XXX	XXXX	0	0	2
Bryozoa							
Tricellaria ternata	1	0	0	0	n.d.	n.d.	–
	2	0	0	0	0	0	0
Eucratea loricata	1	0	0	0	0	0	0
Crisiella sp.	1	0	XX	0	0	0	1
Alcyonidium gelatinosum	1	0	X	X	n.d.	n.d.	–
	2	0	XXX	X	0	0	2
Gastropoda							
Egg mass of *Natica*sp.	1	0	0	0	0	0	0
Nudibranchia							
Dendronotus frondosus	1	0	0	0	0	0	0
Flabellina salmonacea	1	0	X	X	0	0	2
Ascidiacea							
Halocynthia pyriformis	1	0	0	X	0	0	1
Fivefold	1	0	XX	n.d.	0	XX	–
Styela spp.	1	0	0	0	0	0	0
Fivefold	1	0	X	0	0	0	1
Synoicum turgens	1	0	0	0	0	0	0
∑extracts with inhibiting effect		1	7	5	1	2	

Table 4 Inhibition of bacterial growth by crude extract, fractions and pure compounds of the sponge *Haliclona viscosa* and the ascidian *Halocynthia pyriformis*. Radius of inhibition zone: *0* no effect; *X* 0–1 mm (weak inhibition); *XX* >1–3 mm (moderate inhibition); *XXX* >3–7 mm (strong inhibition); *XXXX* >7–15 mm (very strong inhibition). The number of replicates is given in *parentheses*

Extract	Bacterial strain					∑ strains inhibited
	A	B	C	D	E	
Haliclona viscosa						
Crude extract	XXX (4)	XXXX (1)	XXXX (1)	XXX (4)	XXX (4)	5
n-hexane	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0
n-BuOH/EtOHc	XX (5)	XXX (2)	XX (1)	XXX (6)	XXX (5)	5
Water	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0
X2	XX (3)	XX (1)	0 (1)	XX (3)	XXX (3)	4
X3	0 (3)	XXXX (1)	0 (1)	0 (4)	XXX (3)	2
X4	0 (3)	XXXX (1)	X (1)	0 (4)	XXX (3)	3
X2-X3-X4	0 (3)	n.d.	n.d.	0 (3)	XXX (2)	–
X2-X3	X (2)	n.d.	n.d.	0 (2)	XXX (2)	–
X2-X4	X (2)	n.d.	n.d.	0 (2)	XXX (2)	–
X3-X4	0 (2)	n.d.	n.d.	0 (2)	X (2)	–
Halocynthia pyriformis						
Natural concentration						
Crude extract	0 (1)	0 (1)	X (1)	0 (1)	0 (1)	1
n-hexane	n.d.	0 (1)	0 (1)	0 (1)	0 (1)	0
n-BuOH	n.d.	0 (1)	0 (1)	0 (1)	0 (1)	0
EtOAc	n.d.	X (1)	0 (1)	0 (1)	0 (1)	1
Water	n.d.	0 (1)	0 (1)	0 (1)	0 (1)	0
Fivefold natural concentration						
Crude extract	0 (1)	XX (1)	n.d.	0 (1)	XX (1)	–
n-hexane	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0
n-BuOH	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0
EtOAc	0 (1)	XX (1)	X (1)	0 (1)	0 (1)	2
Water	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0

antibacterial activity against non-marine pathogens does not necessarily indicate similar activity against marine bacteria (Bergquist and Bedford 1978; Thompson et al. 1985). Bacterial strains in the present study were isolated from non-living, biologically inert substrates, since bacteria associated with invertebrates may have developed resistance to host metabolites as a result of co-evolution. Kelman et al. (1998) found no activity of coral crude extracts against bacteria isolated from coral tissue and surface mucus; however, two bacterial strains isolated from sponge tissue were the most sensitive test strains among six genera of marine bacteria against sponge extracts (Newbold et al. 1999). Therefore, the use of bacteria isolated from the same organism that is tested for antibacterial activity may lead to over- or under-estimation of the antibiotic potential.

Among the five bacterial strains used in the present study, strains B (Gram-positive) and C (Gram-negative) were most frequently inhibited. Similar to results from Jensen et al. (1996), who found indication that fast- and slow-growing bacteria had different susceptibilities to antibiotic extracts, these two obviously sensitive strains B and C were growing slower compared to the other three bacterial strains.

Antimicrobial activity

Knowledge of the antimicrobial activity in marine invertebrates is not distributed evenly among the taxonomic groups, making geographical comparisons within taxa more difficult. While extensive literature exists on sponges (Burkholder and Ruetzler 1969; Newbold et al. 1999) and gorgonian corals (Kim 1994; Jensen et al. 1996), little is known about bryozoans (Walls et al. 1993), and even less about actinians. However, results from the present study indicate that only a small portion of Arctic invertebrates is chemically defended against bacteria compared to species from lower latitudes and also from Antarctica. McClintock and Gauthier (1992), for example, found 64% of 17 Antarctic sponges to be at least weakly active against marine bacteria. In New Zealand, 76% of 30 sponges inhibited growth of marine bacteria (Bergquist and Bedford 1978), 48% of 33 Carribean sponges had antimicrobial activity (Newbold et al. 1999), and 32% of 28 Mediterranean sponges tested against marine bacteria and yeasts were active (Amade et al. 1987). Of the 18 crude extracts tested in this study, 6 (33%) caused noticeable inhibition of growth of at least 1 of the 5 bacterial strains at natural extract concentration. However, we tested several different taxonomic groups and only four of the extracts originated from sponges. Of these sponges, one (25%) showed antimicrobial activity.

In the literature, there has been considerable discussion about which size of inhibition zone reliably indicates antibiotic activity. For example, Bergquist and Bedford (1978) ignored all zones of inhibition less than 1 mm, and Jensen et al. (1996) regarded extracts as possessing antibacterial activity only if at least two replicates produced zones >5 mm. Other authors have considered any zone of growth inhibition, independent of its size, as an indication of antimicrobial activity (Thompson et al. 1985; Kelman et al. 1998). We, too, recorded zones of any size as positive results, since the diameter of the zone is partially related to the ability of metabolites to diffuse into the agar, and lipid-soluble compounds will often diffuse into the agar less well than more polar compounds (Walls et al. 1993). However, there is no doubt that small zones of inhibition need to be interpreted with caution (Jenkins et al. 1998). Physical and chemical characteristics of the extract such as viscosity or pH can inhibit growth of bacteria and primary metabolites can also exhibit weak inhibitory effects when tested at high concentrations (Jensen et al. 1996; Jenkins et al. 1998). In our study, a few extracts (*Flabellina salmonacea*, *Halocynthia pyriformis*, *Styela* spp.) caused exclusively very small inhibition zones, and while the antimicrobial activity of *H. pyriformis* was confirmed through experiments with increased extract concentrations, further experiments with extracts from *Styela* spp. and *F. salmonacea* are still lacking. If small inhibition zones were to be disregarded, then only 22% of the investigated species show moderate or high inhibition of at least one bacterial strain at natural extract concentrations.

The crude extract of the sponge *Haliclona viscosa* exhibited considerably stronger inhibition of bacteria and affected more bacterial strains than any other extract tested. The strong activity against a variety of different bacterial strains suggests broad-spectrum active compounds although Newbold et al. (1999) mentioned for Caribbean sponges that broad-spectrum antibacterial agents are uncommon. Bioassay-guided fractionation of the crude extract revealed that the strong activity in *Haliclona viscosa* was caused by compounds of the *n*-BuOH/EtOAc fraction. However, none of the purified compounds (X2, X3, X4) alone repeated the effect of the *n*-BuOH/EtOAc fraction. The effect of X2, a compound that also deterred feeding of sympatric amphipods (H. Lippert, K. Iken, C. Volk, M. Köck, E. Rachov, unpublished work), was most comparable to the effects of the crude extract. Surprisingly, the only strain not or only weakly inhibited by the three pure compounds was strain C, which was one of the two more sensitive strains in all other experiments. The various combinations of compounds had similarly strong effects as the pure compounds (Table 4) but no additive or synergistic effects were observed to cause the strong effect of the crude extract. Newbold et al. (1999) also found inhibition zones produced by crude extracts to be greater than those of purified compounds, and suggested that the crude extract may contain additional minor compounds that were not isolated. Furthermore, extraction procedures may lead to a certain loss of compound mass.

Several species of the genus *Haliclona* from tropical and Antarctic regions are reported to contain

biologically active compounds (e.g. McClintock 1987; Baker et al. 1988; Fusetani et al. 1989; Bakus et al. 1994; Jaspars et al. 1994; Charan et al. 1996; Clark et al. 1998; Harrison 1999; Brown et al. 2001). While extracts of *Haliclona cinerea* from California and *Haliclona mediterranea* from the Mediterranean were highly active against bacteria (Thompson et al. 1985; Amade et al. 1987), other species of this genus have been shown to be inactive or only weakly active against micro-organisms (*Haliclona heterofibrosa*, Bergquist and Bedford 1978; *Haliclona* sp., McClintock and Gauthier 1992). From the two species of the genus *Haliclona* (*Haliclona viscosa*, *Haliclona rosea*) occurring in the sub-Arctic Kongsfjord, we found only the extract of *Haliclona viscosa* to possess antibacterial activity. A similar difference in activity was found for antifeeding activity against amphipods in extracts of these two species (H. Lippert, K. Iken, C. Volk, M. Köck, E. Rachov, unpublished work).

In the context of evolutionary questions about bioactive natural compounds and the adaptive influence of environmental and biological factors versus genetically fixed traits, it is an interesting observation that the two closely related species of *Haliclona* in Kongsfjord show very different biological activity. This could suggest that environmental conditions are less important than species-specific traits. In contrast, highly similar activity was found in taxonomically closely related octocorals from opposite polar regions, indicating that environmental factors may be very important. The extract of *G. antarctica*, a close relative to *G. rubiformis* tested in the present study, showed weak (<2 mm) growth inhibition of sympatric bacteria at tissue-level concentration and inhibition was, similar to our results, increased at higher tissue-level concentration (4–6 mm) (Slattery et al. 1995). Both *G. antarctica* and *G. rubiformis* contain bioactive compounds that also deter feeding of sympatric predators (Slattery and McClintock 1995; H. Lippert, K. Iken, C. Volk, M. Köck, E. Rachov, unpublished work). Biological activity of extracts can also differ within populations of the same species from different locations. The sponge *Suberites ficus* from Kongsfjord did not show antimicrobial activity, but extracts of the same species from southern Britain were active against a marine and a non-marine bacterial strain (Dyrynda 1985). Direct comparisons between these different studies, however, should be considered cautiously because of the variety of methods used and the different bacterial strains employed. This variety within antimicrobial activity across species and geographic regions shows that evolutionary considerations within chemical ecology will need to be much more rigorously tested before it may be possible to unravel any patterns.

Observations in nature

Deterring microbial attachment and inhibiting microbial growth may be strategies for limiting the establishment of later successional fouling stages through the prevention of surface conditioning by early successional bacteria (Thompson et al. 1985; Davis et al. 1989; Wahl 1989; Wahl et al. 1994). From our own underwater observations, we saw that in nature the investigated invertebrates were fouled to different degrees with epizoids, although we could not collect quantitative data. In some cases, a relationship between the degree of fouling and the antimicrobial activity seems to exist. For example, the soft coral *G. rubiformis* and the ascidian *Halocynthia pyriformis* were never fouled and also exhibited antibacterial activity. The ascidian *Styela* spp. and the bryozoans *Tricellaria ternata* and *Eucratea loricata* were heavily fouled and did not inhibit bacterial growth. However, there are several non-fouled species that also did not show any antibacterial activity in our experiments, like the actinians *Hormatia nodosa*, *Urticina asiatica* and *U. eques*, and the sponge *Suberites ficus*. There may be mechanisms other than secondary metabolites responsible for antifouling properties in *H. nodosa* and *U. eques*, for example, mechanical or physical defences like tissue sloughing (Barthel and Wolfrath 1989) or mucus secretion (Krupp 1985).

Similar observations were made if we compare the number of bacterial strains that could be cultured from swabbing similarly sized surface areas of invertebrates. This is by no means a quantitative measure of the actual degree of microbial fouling and it only refers to those bacterial strains that grow under culture conditions. We found, however, that the actinians *Hormathia nodosa* and *U. eques* yielded very few bacteria in culture while some other species, e.g. the ascidian *Styela* spp., that were overgrown with fouling macro-organisms also yielded a large number of cultivable surface bacteria.

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